

# Influence of GABA<sub>A</sub> Receptor $\alpha$ Subunit Isoforms on the Benzodiazepine Binding Site

Benjamin P. Lüscher<sup>1</sup>, Roland Baur<sup>1</sup>, Maurice Goeldner<sup>2</sup>, Erwin Sigel<sup>1\*</sup>

<sup>1</sup> Institute of Biochemistry and Molecular Medicine, University of Bern, Bern, Switzerland, <sup>2</sup> Laboratoire de Conception et Application de Molécules Bioactives, Unité Mixte de Recherche CNRS, Faculté de Pharmacie, Université de Strasbourg, Illkirch, France

## Abstract

Classical benzodiazepines, such as diazepam, interact with  $\alpha_x\beta_2\gamma_2$  GABA<sub>A</sub> receptors,  $x=1, 2, 3, 5$  and modulate their function. Modulation of different receptor isoforms probably results in selective behavioural effects as sedation and anxiolysis. Knowledge of differences in the structure of the binding pocket in different receptor isoforms is of interest for the generation of isoform-specific ligands. We studied here the interaction of the covalently reacting diazepam analogue 3-NCS with  $\alpha_1S204C\beta_2\gamma_2$ ,  $\alpha_1S205C\beta_2\gamma_2$  and  $\alpha_1T206C\beta_2\gamma_2$  and with receptors containing the homologous mutations in  $\alpha_2\beta_2\gamma_2$ ,  $\alpha_3\beta_2\gamma_2$ ,  $\alpha_5\beta_{1/2}\gamma_2$  and  $\alpha_6\beta_2\gamma_2$ . The interaction was studied using radioactive ligand binding and at the functional level using electrophysiological techniques. Both strategies gave overlapping results. Our data allow conclusions about the relative apposition of  $\alpha_1S204C\beta_2\gamma_2$ ,  $\alpha_1S205C\beta_2\gamma_2$  and  $\alpha_1T206C\beta_2\gamma_2$  and homologous positions in  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_5$  and  $\alpha_6$  with C-atom adjacent to the keto-group in diazepam. Together with similar data on the C-atom carrying Cl in diazepam, they indicate that the architecture of the binding site for benzodiazepines differs in each GABA<sub>A</sub> receptor isoform  $\alpha_1\beta_2\gamma_2$ ,  $\alpha_2\beta_2\gamma_2$ ,  $\alpha_3\beta_2\gamma_2$ ,  $\alpha_5\beta_{1/2}\gamma_2$  and  $\alpha_6\beta_2\gamma_2$ .

**Citation:** Lüscher BP, Baur R, Goeldner M, Sigel E (2012) Influence of GABA<sub>A</sub> Receptor  $\alpha$  Subunit Isoforms on the Benzodiazepine Binding Site. PLoS ONE 7(7): e42101. doi:10.1371/journal.pone.0042101

**Editor:** Stefan Strack, University of Iowa, United States of America

**Received:** February 23, 2012; **Accepted:** July 2, 2012; **Published:** July 27, 2012

**Copyright:** © 2012 Lüscher et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was supported by the Swiss National Foundation (<http://www.snf.ch>) grant 31003A\_132806/1. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

\* E-mail: [erwin.sigel@ibmm.unibe.ch](mailto:erwin.sigel@ibmm.unibe.ch)

## Introduction

Benzodiazepines are widely used drugs. They exert sedative/hypnotic, anxiolytic, muscle relaxant, and anticonvulsant effects. Benzodiazepines are safe and effective in short term treatments even if some side effects as anterograde amnesia have been reported.

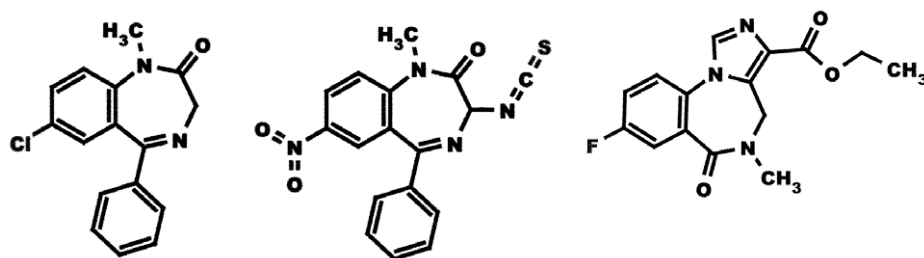
Benzodiazepines act at the major inhibitory neurotransmitter receptor, the  $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>) receptor. The GABA<sub>A</sub> receptors are composed of five subunits surrounding a central chloride ion selective channel [1–4]. A variety of subunit isoforms of the GABA<sub>A</sub> receptor has been cloned, leading to a multiplicity of receptor subtypes [1,2,5,6]. The major receptor isoform in mammalian brain consists of  $\alpha_1$ ,  $\beta_2$ , and  $\gamma_2$  subunits [6,7]. Different approaches have indicated a  $2\alpha:2\beta:1\gamma$  subunit stoichiometry for this receptor [8–13] with a subunit arrangement  $\gamma\beta\alpha\beta\alpha$  anti-clockwise as seen from the synaptic cleft [11–13]. The classical benzodiazepine diazepam binds with high affinity and positively modulates recombinant  $\alpha_1\beta_x\gamma_2$  ( $x=1, 2, 3$ ),  $\alpha_2\beta_x\gamma_2$ ,  $\alpha_3\beta_x\gamma_2$  and  $\alpha_5\beta_x\gamma_2$  GABA<sub>A</sub> receptors. The high affinity binding site has been located between the  $\alpha$  and  $\gamma$  subunit and is homologous to the agonist binding site located between the  $\beta$  and  $\alpha$  subunit [14,15]. Even if it is very common in the field to discuss e.g. “ $\alpha_1$  receptors”, there is good evidence for the fact that many GABA<sub>A</sub> receptors contain two different  $\alpha$  subunit isoforms (e.g. [16]). Exclusively the  $\alpha$  subunit adjacent to the  $\gamma$  subunit defines the nature of the benzodiazepine site [17].

It has been demonstrated that the residue H101 within the  $\alpha_1$  subunit [18] and the homologous residues  $\alpha_2$ H101,  $\alpha_3$ H126 and

$\alpha_5$ H105 are crucial for diazepam potentiation [19]. In  $\alpha_4$  and  $\alpha_6$  containing receptors the homologous residue is an arginine rendering these receptors insensitive to classical benzodiazepines [18,20,21]. Replacement of arginine by histidine in  $\alpha_4$  and  $\alpha_6$  confers benzodiazepine sensitivity [18] and replacement of histidine by arginine in  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$  and  $\alpha_5$  abolishes modulation by diazepam [18,19].

Pharmacological and behavioral studies of knock-in mice in which the relevant histidine residue has been mutated to an arginine, have led to correlations between the  $\alpha$  subunit isoform adjacent to the  $\gamma$  subunit and several of the behavioral effects mediated by benzodiazepines. These studies have revealed that GABA<sub>A</sub> receptors containing an  $\alpha_1$  subunit in this position mediate the sedative, the anterograde amnesic and partly the anticonvulsive effects of diazepam [22,23]. GABA<sub>A</sub> receptors containing an  $\alpha_2$  subunit in this position mediate the anxiolytic effect and the myorelaxant effect [24,25]. GABA<sub>A</sub> receptors containing either an  $\alpha_3$  or an  $\alpha_5$  subunit adjacent to the  $\gamma$  subunit contribute to the myorelaxant actions of benzodiazepines [24,26,27].

Diazepam is arranged in the binding pocket of  $\alpha_1\beta_2\gamma_2$  receptors such as to allow interaction of a reactive group replacing the –Cl atom with a reactive residue in place of  $\alpha_1$ H101 [28–31] and interaction of a reactive group attached to the 3'-atom with a reactive residues in place of  $\alpha_1$ S205 or  $\alpha_1$ S206 [32]. It has also been shown that a reactive residue in place of the –Cl atom interacts with a reactive residue in place of the histidine homologous to  $\alpha_1$ H101 in  $\alpha_2$ ,  $\alpha_3$  and the



**Figure 1. Chemical structures of diazepam, 3-NCS and Ro15-1788.**

doi:10.1371/journal.pone.0042101.g001

corresponding arginine residue in  $\alpha_6$  but not to the histidine residue in  $\alpha_5$  [33].

In addition to the high affinity binding site for benzodiazepines, two low affinity binding sites were identified; one has been described to be located in the lipid bilayer [34] and the second at the  $\alpha/\beta$  subunit interface [33,35].

We used the proximity-accelerated chemical reaction approach to further characterize the architecture of the benzodiazepine binding site in different receptor isoforms. GABA<sub>A</sub> receptor residues thought to reside in the site were individually mutated to cysteine and combined with a modified benzodiazepine molecule carrying a substituent reactive with cysteine at the 3' atom (3-NCS, Fig. 1). Direct apposition of target carbon atom of the NCS group and the reactive  $-S^-$  group of cysteine is expected to lead to a covalent reaction.

We studied interaction of 3-NCS compound with amino acid residues 204, 205 and 206 in  $\alpha_1\beta_2\gamma_2$  receptors and the homologous residues in  $\alpha_2\beta_2\gamma_2$ ,  $\alpha_3\beta_2\gamma_2$ ,  $\alpha_5\beta_1\gamma_2$  and  $\alpha_6\beta_2\gamma_2$  (Fig. 2), using radioactive ligand binding studies at receptors expressed in HEK-cells and electrophysiological studies, using the two electrode voltage clamp technique at receptors expressed in *Xenopus laevis* oocytes. In each receptor isoform  $\alpha_x\beta_2\gamma_2$  ( $x = 1, 2, 3, 5, 6$ ), we found a different interaction pattern of the corresponding  $\alpha$  subunit with the cysteine reactive compound. This indicates a difference in shape of the benzodiazepine binding site in the region of the 3' atom of diazepam in different receptor isoforms. We further observed disruption of benzodiazepine binding site in  $\alpha_1G207C\beta_2\gamma_2$  and  $\alpha_2G207C\beta_2\gamma_2$  receptors.

## Methods

### Construction of the Mutated Receptor Subunits

The mutant subunits  $\alpha_1S204C$ ,  $\alpha_1S205C$ ,  $\alpha_1T206C$ ,  $\alpha_1G207C$ ,  $\alpha_2S204C$ ,  $\alpha_2S205C$ ,  $\alpha_2T206C$ ,  $\alpha_2G207C$ ,  $\alpha_3S229C$ ,  $\alpha_3S230C$ ,  $\alpha_3T231C$ ,  $\alpha_5T208C$ ,  $\alpha_5S209C$ ,  $\alpha_5T210C$ ,  $\alpha_6S203C$ ,  $\alpha_6N204C$  and  $\alpha_6T205C$  were prepared using the QuikChange<sup>TM</sup> mutagenesis kit (Stratagene). For cell transfection, the cDNAs were subcloned into the polylinker of pBC/CMV [36]. This expression vector allows high-level expression of a foreign gene under control of the cytomegalovirus promoter.

### The Cysteine-reactive Compound

The synthesis of the cysteine-reactive compound 3-NCS (Fig. 1) has been described before [32].

### Transfection of GABA<sub>A</sub> Receptors in HEK293 Cells and Membrane Preparation

cDNAs coding for the  $\alpha_x$  ( $x = 1, 2, 3, 6$ ),  $\beta_2$ , and  $\gamma_2S$  subunits DNA (20  $\mu$ g : 20  $\mu$ g : 20  $\mu$ g) per 9 cm diameter dish were transfected in human embryonic kidney (HEK) 293 cells

(American Type of Culture Collection, MD, USA, CRL 1573) using the calcium phosphate precipitation technique [37]. For  $\alpha_5$  containing receptors  $\beta_2$  was replaced by  $\beta_1$ . This resulted in higher expression levels. Culturing of cells and membrane preparation were done as described before [30].

### Radioactive Ligand Binding Assay

The properties of the recombinant mutant receptors were only estimated. For this affinity estimate, membranes were re-suspended in phosphate buffer using a Teflon homogenizer. They were incubated in a total volume of 360  $\mu$ l for 1 h on ice in the presence of [<sup>3</sup>H]Ro15-1788 (78.6 Ci/mmol; PerkinElmer Life Sciences). The final protein concentration was 0.1–1 mg of protein/ml. Total binding was measured at 0.5 and 5 nM [<sup>3</sup>H]Ro15-1788. Nonspecific binding was determined under the same condition but in the presence of 100  $\mu$ M unlabeled Ro15-1788 and amounted to less than 10% of total binding, except for  $\alpha_2S206C\beta_2\gamma_2$  and  $\alpha_5T208C\beta_2\gamma_2$  where it amounted to 10–17%. Expression levels of  $\alpha_6\beta_2\gamma_2$  receptors were estimated with [<sup>3</sup>H]Ro15-4513. Membranes were collected by rapid vacuum filtration on GF/C filters. After three washing steps (3 sec each) with 5 ml of phosphate buffer, the filter-retained radioactivity was determined by liquid scintillation counting.

### Detection of a Covalent Reaction

As detailed in previous work [28,30,33] this procedure included three steps: incubation of membranes expressing recombinant wild type or mutant receptors with the reactive agent followed by extensive washing of the membranes in order to remove non-reacted compound and a radioactive ligand binding assay to determine residual binding. No covalent reaction would result in 100% residual binding, and 100% covalent reaction would result in 0% residual binding.

$\alpha_1$	199	SGIVQ <u>ST</u> GEYVVM	212
$\alpha_2$	199	KETIK <u>S</u> STGEYTVM	212
$\alpha_3$	224	TEIIR <u>S</u> STGEYVVM	237
$\alpha_4$	200	SETIKSITGEYIVM	213
$\alpha_5$	203	TENIS <u>T</u> STGEYTIM	216
$\alpha_6$	198	SETIKSNTGEYVIM	211

**Figure 2. Alignment of Loop C of the  $\alpha$  subunits.** The underlined residues were individually mutated to cysteine. The bold residues react covalently with 3-NCS.  $\alpha_4$  was not investigated and is only shown for comparison.

doi:10.1371/journal.pone.0042101.g002

Briefly, the membranes were re-suspended in phosphate buffer (100 mM KCl, 10 mM  $\text{KH}_2\text{PO}_4$ , 0.1 mM EDTA, pH 7.4) using a Glass/Teflon homogenizer. 0.1–1.0 mg/mL of protein were incubated in a total volume of 360  $\mu\text{L}$  with either 10  $\mu\text{M}$  (determination of degree of covalent reaction) or several concentrations of 3-NCS for 30 min on ice. Membranes were collected with rapid filtration on a round 7 mm diameter glass fiber filter (GF/C; Whatman) that was placed on a round 24 mm diameter glass fiber filter (GF/C; Whatman), both pre-washed with phosphate buffer. The reaction of 3-NCS with the receptor was stopped by washing of the filters six times with 5 mL phosphate buffer, each. The small filters with the deposited membranes were incubated in 0.12 mL phosphate buffer containing 5 nM [ $^3\text{H}$ ]Ro15–1788. After 30 min the 7 mm filter was placed on a 24 mm filter and washed six times with 5 mL phosphate buffer each. Radioactivity was determined by liquid scintillation counting. Non-specific binding was determined in the presence of 100  $\mu\text{M}$  Ro 15–1788. In control experiments, washing efficiency was estimated by placing radioactivity on the small filter. More than 99.95% of the radioactivity was removed (not shown).

Concentration response curves for 3-NCS were fitted with the equation  $C(c) = C_{\text{max}}/(1+(\text{EC}_{50}/c))$ , where  $c$  is the concentration of 3-NCS,  $\text{EC}_{50}$  the concentration of 3-NCS where half maximal covalent reaction was observed,  $C_{\text{max}}$  is the maximal extent of the covalent reaction and  $C$  the measured extent of the covalent reaction.

### Expression of GABA<sub>A</sub> Receptors in Xenopus Oocytes

Capped cRNAs were synthesized (Ambion, Austin, TX, USA) from the linearized plasmids with a cytomegalovirus promoter (pCMVvectors) containing the different subunits, respectively. A poly-A tail of about 400 residues was added to each transcript using yeast poly- A polymerase (United States Biologicals, Cleveland, OH, USA). The concentration of the cRNA was quantified on a formaldehyde gel using Radiant Red stain (Bio-Rad) for visualization of the RNA. Known concentrations of RNA ladder (Invitrogen) were loaded as standard on the same gel. cRNAs were precipitated in ethanol/isoamylalcohol 19: 1, the dried pellet dissolved in water and stored at  $-80^\circ\text{C}$ . cRNA mixtures were prepared from these stock solutions and stored at  $-80^\circ\text{C}$ . *Xenopus laevis* oocytes were prepared, injected and defolliculated as described previously [38,39]. They were injected with 50 nL of the cRNA solution containing wild type or mutated  $\alpha_1$  or  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_5$ ,  $\alpha_6$  and wild type  $\beta_2$  and  $\gamma_2$  subunits at a concentration of 10 nM : 10 nM : 50 nM [40] and then incubated in modified Barth's solution at  $+18^\circ\text{C}$  for at least 24 h before the measurements.

### Functional Characterization of the GABA<sub>A</sub> Receptors

Currents were measured using a modified two-electrode voltage clamp amplifier Oocyte clamp OC-725 (Warner Instruments) in combination with a XY-recorder (90% response time 0.1s) or digitized at 100 Hz using a PowerLab 2/20 (AD Instruments) using the computer programs Chart (ADInstruments GmbH, Spechbach, Germany). Tests with a model oocyte were performed to ensure linearity in the larger current range. The response was linear up to 15  $\mu\text{A}$ .

Electrophysiological experiments were performed by using the two-electrode voltage clamp method at a holding potential of  $-80\text{ mV}$ . The perfusion medium contained 90 mM NaCl, 1 mM KCl, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , and 5 mM Na-HEPES (pH 7.4) and was applied by gravity flow 6 ml/min. The perfusion medium was applied through a glass capillary with an inner diameter of 1.35 mm, the mouth of which was placed about

0.4 mm from the surface of the oocyte. Allosteric modulation via the benzodiazepine site was measured at a GABA concentration eliciting 2–5% of the maximal GABA current amplitude. GABA was applied for 20 s alone or in combination with allosteric compound. 6 ml of the covalent reacting compound was applied to the oocyte and incubated for 3 min while stopping the flow of the perfusion medium. Modulation of GABA currents was expressed as  $(I_{(\text{modulator} + \text{GABA})}/I_{\text{GABA}} - 1) * 100\%$ . The perfusion system was cleaned between drug applications by washing with DMSO to avoid contamination.

## Results

We wanted to derive information on part of the benzodiazepine binding pockets in different GABA<sub>A</sub> receptor isoforms. For this purpose, we characterized the covalent interaction of a benzodiazepine-like compound with  $\alpha_x\beta_{1/2}\gamma_2$  ( $x = 1, 2, 3, 5, 6$ ) receptors containing a cysteine mutation in residues  $\alpha_1\text{S204C}$ ,  $\alpha_1\text{S205C}$  and  $\alpha_1\text{T206C}$  and homologous positions in  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_5$  and  $\alpha_6$ . The  $\beta_2$  subunit was used throughout except for expression of  $\alpha_5$  containing receptors in HEK-cells where  $\beta_1$  was used instead to achieve higher expression levels.  $\alpha_1\text{G207C}$  and the homologous mutation  $\alpha_2\text{G207C}$  were also prepared. These mutated subunits did not confer to the expressed receptors high affinity [ $^3\text{H}$ ]Ro 15–1788 or [ $^3\text{H}$ ]flunitrazepam binding nor functional modulation by diazepam (not shown). This indicates that mutation in this position disrupts the binding site for benzodiazepines.

### The Cysteine Reactive Compound

We used a modified nitrazepam molecule carrying a cysteine reactive isothiocyanate substituent at the C-3 carbon (3-NCS; Fig. 1). 3-NCS was able to displace [ $^3\text{H}$ ]Ro15–1788 or [ $^3\text{H}$ ]flunitrazepam from wild-type  $\alpha_1\beta_2\gamma_2$  receptors. The  $K_i$  values were  $340 \pm 16\text{ nM}$  and  $240 \pm 55\text{ nM}$  [32], respectively. The  $K_i$  values for displacement of [ $^3\text{H}$ ]Ro 15–1788 by 3-NCS in  $\alpha_2\beta_2\gamma_2$  were  $1550 \pm 250\text{ nM}$  ( $n = 3$ ) and in  $\alpha_5\beta_1\gamma_2$  receptors  $9840 \pm 1480\text{ nM}$  ( $n = 3$ ). The determination of  $K_i$  values was based on the  $K_d$  values of  $2.1 \pm 0.5\text{ nM}$  and  $1.5 \pm 1.2\text{ nM}$ , respectively. The covalent reaction of 3-NCS with  $\alpha_1\text{m}\beta_2\gamma_2$  ( $\text{m} = \text{S205C, T206C}$ ) receptor at the binding and functional level has previously been described [32].

### Binding Properties of the GABA<sub>A</sub> Receptor Carrying a Cysteine Point Mutation

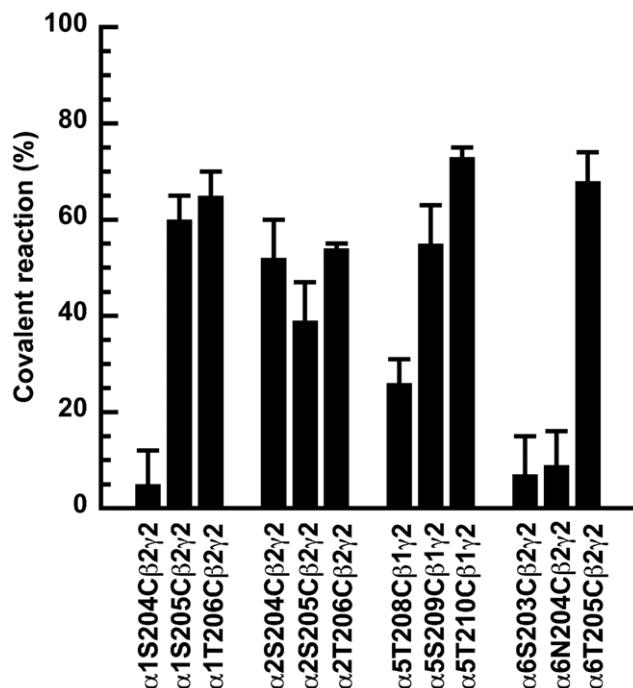
The homologous residues to  $\alpha_1\text{S204}$ ,  $\alpha_1\text{S205}$  and  $\alpha_1\text{T206}$  in  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_5$  and  $\alpha_6$  were mutated individually to cysteine.  $\alpha_1$  and  $\alpha_2$  were expressed in combination with  $\beta_2$  and  $\gamma_2$  subunits,  $\alpha_5$  for reasons mentioned above together with  $\beta_1$  and  $\gamma_2$  subunits. All these mutated  $\alpha_m\beta_2\gamma_2$  receptors bound [ $^3\text{H}$ ]Ro 15–1788 with an estimated affinity between 0.08 and 7.5 nM (data not shown). No specific binding was detected in  $\alpha_3$  containing receptors (data not shown).  $\alpha_6\text{T205C}\beta_2\gamma_2$  bound [ $^3\text{H}$ ]Ro15–4513 with an estimated affinity 4 nM.

### Covalent Reaction of 3-NCS at the Binding Level

First we tested reactivity of 3-NCS using a radioactive ligand binding assay. 3-NCS is expected to first occupy its binding site reversibly. Upon proper apposition of the  $-\text{SH}$  group of the cysteine from the mutated receptors with the  $-\text{C}$  atom of the NCS group from the 3-NCS compound, this is followed by covalent reaction. This reaction was determined at 10  $\mu\text{M}$  concentration of 3-NCS. Preliminary experiments showed that at this concentration and at 1  $\mu\text{M}$  3-NCS, covalent reaction was reaching a maximum within 15 sec (not shown). Covalent reaction did not reach

completion. This observation was made before and has been interpreted to indicate covalent reaction at a low affinity benzodiazepine binding site at the  $\alpha/\beta$  subunit interface, which prevents covalent reaction on the classical benzodiazepine binding site [33]. Alternatively or in addition, the reactive compound may be consumed in non-specific reactions. Non-covalently reacted compound was removed by filtration (see methods). A covalent reaction of 3-NCS with a mutated cysteine residue is expected to prevent reversible binding of the [ $^3\text{H}$ ]Ro15-1788. If no covalent reaction occurs, the binding site should still be available for reversible binding.  $\alpha_5$  containing receptors were expressed together with  $\beta_1$  instead of  $\beta_2$ .  $\alpha_3$  containing receptors did not express in HEK293-cells. As  $\alpha_6$  subunit containing receptors do not bind [ $^3\text{H}$ ]Ro15-1788 with high affinity, we used [ $^3\text{H}$ ]Ro15-4513 instead.

Figure 3 compares the percentage of covalent reaction, in mutated  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_5$  and  $\alpha_6$  containing receptors. For the  $\alpha_1\beta_2\gamma_2$  receptor isoform cysteine mutation in residue S205 and T206 resulted in covalent reaction, while S204C did not covalently react with 3-NCS [32]. For  $\alpha_2\beta_2\gamma_2$  receptor isoform the cysteine mutated residues homologous to  $\alpha_1$ S204,  $\alpha_1$ S205 and  $\alpha_1$ T206 showed covalent reaction amounting to  $52\pm 8\%$  ( $n=3$ ),  $39\pm 8\%$  ( $n=3$ ) and  $54\pm 1\%$  ( $n=3$ ). For  $\alpha_5\beta_1\gamma_2$  receptors the cysteine mutated receptors homologous to  $\alpha_1$ S204,  $\alpha_1$ S205 and  $\alpha_1$ T206 reacted covalently amounting to  $26\pm 5\%$  ( $n=3$ ),  $54\pm 8\%$  ( $n=3$ ) and  $73\pm 2\%$  ( $n=3$ ). In the  $\alpha_6\beta_2\gamma_2$  receptor



**Figure 3. Covalent reaction at the binding level.** Extent of covalent reaction of 3-NCS at  $\alpha_1$ S204C $\beta_2\gamma_2$ ,  $\alpha_1$ S205C $\beta_2\gamma_2$ ,  $\alpha_1$ T206C $\beta_2\gamma_2$  mutant GABA<sub>A</sub> receptors and homologous receptors formed with  $\alpha_2$ ,  $\alpha_3$ , and  $\alpha_6$ .  $\alpha_5$  was expressed together with  $\beta_1$  and  $\gamma_2$  subunits for reasons mentioned in the results section. Receptors were expressed in HEK-293 cells, membranes harvested and exposed to 10  $\mu\text{M}$  3-NCS. After incubation, the residual 3-NCS was removed by filtration. [ $^3\text{H}$ ]Ro15-1788 was used as radioactive ligand to determine the residual binding. Covalent binding is expressed as 100% minus % residual binding. Data are shown as mean  $\pm$ SD for three experiments each (triplicates of each point in each experiment). doi:10.1371/journal.pone.0042101.g003

isoform only the cysteine mutation homologous to  $\alpha_1$ T206 reacted covalently with 3-NCS amounting to  $68\pm 6\%$  ( $n=3$ ).

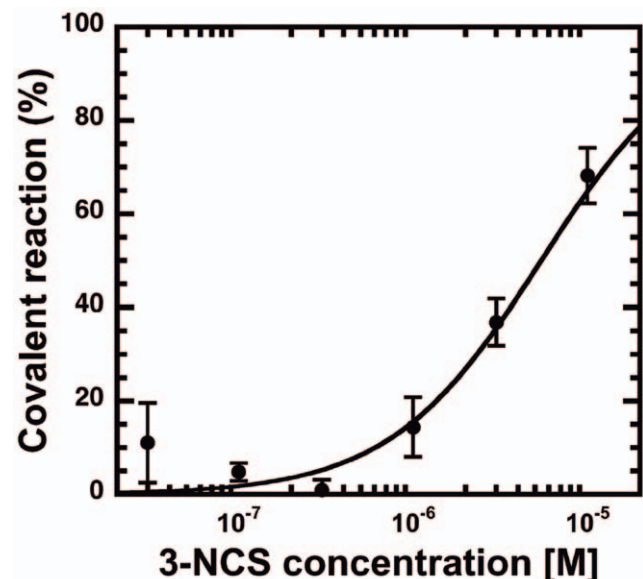
### Concentration Dependence of the Covalent Reaction

We next investigated the concentration dependence of the covalent reaction. Mutated receptors were exposed for 30 min to different concentrations of 3-NCS. Figure 4 documents such a concentration dependence in  $\alpha_6$ T205C $\beta_2\gamma_2$  receptors. The concentration dependence was also determined in mutated  $\alpha_1$ S204C $\beta_2\gamma_2$ ,  $\alpha_1$ S205C $\beta_2\gamma_2$ ,  $\alpha_1$ T206C $\beta_2\gamma_2$  and homologous mutations in  $\alpha_2$ ,  $\alpha_5$  and  $\alpha_6$  containing receptors. Table 1 documents the calculated  $\text{EC}_{50}$  values of the covalent reaction, which are between 0.08  $\mu\text{M}$  and 6.6  $\mu\text{M}$ . For  $\alpha_1$ S204C $\beta_2\gamma_2$ ,  $\alpha_6$ S203C $\beta_2\gamma_2$ ,  $\alpha_6$ N204C $\beta_2\gamma_2$  the  $\text{EC}_{50}$  values could not be determined because we could not detect covalent reaction with 3-NCS.

Residue 206 is unique in that all the investigated subunit isoforms do show covalent reaction. In each case the absolute level of the reaction is relatively high (Fig. 3). It should be noted that residue 206 in the  $\alpha_1$  subunit and homologous residues in  $\alpha_2$  and  $\alpha_5$  show a very high apparent affinity in the reaction with 3-NCS while the corresponding residue in  $\alpha_6$  shows a lower apparent affinity (F-test;  $p<0.01$ ). In  $\alpha_1$ ,  $\alpha_2$  and  $\alpha_5$ , the residue identical to or homologous to residue 206 in the  $\alpha_1$  subunit has a lower  $\text{EC}_{50}$  than residue 205 in the corresponding  $\alpha$  subunit (F-test;  $p<0.01$ ). Residue 205 in the  $\alpha_1$  subunit shows a higher apparent affinity in the reaction with 3-NCS than the homologous residue in  $\alpha_2$  (F-test;  $p<0.02$ ).

### Irreversible Reaction of 3-NCS at the Functional Level

We also studied the covalent reaction at the functional level. All wild type receptors failed to show any changes in the current amplitude elicited by GABA, while some mutant receptors did. An example of this experiment is shown in Figure 5a for the



**Figure 4. Concentration dependence at the binding level.** Concentration dependence of the covalent reaction of the 3-NCS at  $\alpha_6$ T205C $\beta_2\gamma_2$  mutant GABA<sub>A</sub> receptors. Extent of covalent reaction was determined at different concentrations of 3-NCS as indicated below Fig. 3. Data are shown as mean  $\pm$ SD for three experiments each (triplicates of each point in each experiment). doi:10.1371/journal.pone.0042101.g004

**Table 1.** Concentration dependence of the covalent reaction at mutant receptors at the binding level.

mutated receptor	mutation homologous to	EC <sub>50</sub> (μM)
α <sub>1</sub> S204Cβ <sub>2</sub> γ <sub>2</sub>		n.r.
α <sub>1</sub> S205Cβ <sub>2</sub> γ <sub>2</sub>		0.63±0.18
α <sub>1</sub> T206Cβ <sub>2</sub> γ <sub>2</sub>		0.082±0.028
α <sub>2</sub> S204Cβ <sub>2</sub> γ <sub>2</sub>	α <sub>1</sub> S204C	3.2±2.3
α <sub>2</sub> S205Cβ <sub>2</sub> γ <sub>2</sub>	α <sub>1</sub> S205C	6.6±5.4
α <sub>2</sub> T206Cβ <sub>2</sub> γ <sub>2</sub>	α <sub>1</sub> S206C	0.19±0.056
α <sub>5</sub> T208Cβ <sub>1</sub> γ <sub>2</sub>	α <sub>1</sub> S204C	0.39±0.21
α <sub>5</sub> S209Cβ <sub>1</sub> γ <sub>2</sub>	α <sub>1</sub> S205C	4.1±2.2
α <sub>5</sub> T210Cβ <sub>1</sub> γ <sub>2</sub>	α <sub>1</sub> S206C	0.45±0.15
α <sub>6</sub> S203Cβ <sub>2</sub> γ <sub>2</sub>	α <sub>1</sub> S204C	n.r. <sup>a</sup>
α <sub>6</sub> N204Cβ <sub>2</sub> γ <sub>2</sub>	α <sub>1</sub> S205C	n.r. <sup>a</sup>
α <sub>6</sub> T205Cβ <sub>2</sub> γ <sub>2</sub>	α <sub>1</sub> S206C	5.4±0.80 <sup>b</sup>

Left column: mutated receptors. Middle column: homology of the mutations to α<sub>1</sub>. Right column EC<sub>50</sub> is given as mean ±SD for three to four experiments where each point in the dose-response curve was determined in triplicates. The receptors were exposed to increasing concentrations of 3-NCS compound for 30 min on ice and extensively washed. Residual binding was determined using [<sup>3</sup>H]Ro15–1788 as radioactive ligand and converted to percentage of binding sites covalently reacted. All wild type receptors showed no covalent reaction. n.r.: no covalent reaction.

<sup>a</sup>[<sup>3</sup>H]15–4513 was used as a radioactive ligand.

doi:10.1371/journal.pone.0042101.t001

α<sub>1</sub>S205Cβ<sub>2</sub>γ<sub>2</sub> mutant receptor. 3-NCS led to an irreversible increase in the current amplitude and the relative stimulation by diazepam was smaller than observed in naïve oocytes (Fig. 5b). We interpret this as evidence for a covalent reaction. Fig. 5c shows the same experiment at the mutant receptor α<sub>1</sub>T204Cβ<sub>2</sub>γ<sub>2</sub>. 3-NCS did not increase the current amplitude and diazepam stimulated to the same extent as in naïve oocytes (Fig. 5d). Clearly, this mutant receptor did not show any covalent reaction. As α<sub>6</sub> containing receptors did not respond to diazepam, we used 1 μM abercarnil instead.

Exposure of mutant receptors α<sub>1</sub>S205Cβ<sub>2</sub>γ<sub>2</sub>, α<sub>1</sub>T206Cβ<sub>2</sub>γ<sub>2</sub>, α<sub>2</sub>T205Cβ<sub>2</sub>γ<sub>2</sub> (mutation homologous to α<sub>1</sub>S205), α<sub>2</sub>T206Cβ<sub>2</sub>γ<sub>2</sub> (α<sub>1</sub>T206), α<sub>5</sub>T231Cβ<sub>2</sub>γ<sub>2</sub> (α<sub>1</sub>T206), α<sub>5</sub>T208Cβ<sub>2</sub>γ<sub>2</sub> (α<sub>1</sub>S204), α<sub>5</sub>T209Cβ<sub>2</sub>γ<sub>2</sub> (α<sub>1</sub>S205), and possibly α<sub>3</sub>S230Cβ<sub>2</sub>γ<sub>2</sub> (α<sub>1</sub>S205) and α<sub>6</sub>T205Cβ<sub>2</sub>γ<sub>2</sub> (α<sub>1</sub>T206), to 3-NCS resulted in an irreversible positive allosteric modulation (Table 2) indicating a covalent reaction, the adduct acting as a positive allosteric modulator. If only a fraction of the receptors react with 3-NCS, diazepam is expected to stimulate the remaining receptors. If 3-NCS has a similar allosteric effect as diazepam the combined stimulations of 3-NCS and diazepam should be the same as stimulation by diazepam alone. This is the case for most mutated receptors studied. Exceptions are α<sub>1</sub>T206Cβ<sub>2</sub>γ<sub>2</sub>, α<sub>3</sub>T231Cβ<sub>2</sub>γ<sub>2</sub> (homologous to α<sub>1</sub>T206), α<sub>5</sub>T208Cβ<sub>2</sub>γ<sub>2</sub> (α<sub>1</sub>S204) and α<sub>5</sub>T210Cβ<sub>2</sub>γ<sub>2</sub> (α<sub>1</sub>T206). In the last two cases, results may be explained if 3-NCS acts at these mutated receptors as a partial modulator and an antagonist, respectively, at the benzodiazepine site. In the first two cases combined stimulation by 3-NCS and diazepam is larger than expected from diazepam alone. The latter seems in both cases reduced by the mutation for unknown reasons. We can only hypothesize that covalent reaction with 3-NCS at the α/β subunit interface may allosterically stimulate the reaction to diazepam. It is interesting to note that at the functional level the identical residues were identified to react covalently as at

the binding level. This in spite of the use of Ro15–1788 to detect covalent reaction at the binding level and the use of diazepam at the functional level. The only exception is α<sub>2</sub>S204C. We have no explanation for this discrepancy. Differences in the experimental conditions used in binding and functional experiments (total lipid content, lipid composition and temperature) may be responsible.

## Discussion

The most common GABA<sub>A</sub> receptors contain two identical or different α subunits [6]. Receptors containing different α subunit isoforms adjacent to the γ subunit mediate different effects of classical benzodiazepines. α<sub>1</sub>, α<sub>2</sub>, α<sub>3</sub> or α<sub>5</sub> are required in this position for the action of classical benzodiazepines. In order to be able to rationally design receptor subtype specific drugs more knowledge on the difference of the interaction of different receptor subtypes with classical benzodiazepines is required.

We aimed at finding differences or similarities in the shape of the benzodiazepine binding site in different receptor isoforms. We used the proximity-accelerated chemical coupling reaction at the binding and functional level. A Cysteine-mutated receptor is combined with a chemical modified cysteine reactive binding site ligand. Identification of a covalent reaction implies apposition of the –S atom of cysteine and the reactive –C atom of the ligand [41]. After previous work in the α<sub>1</sub>H101 region [33], where we found that a NCS group introduced into diazepam at the –Cl position interacts subtly different with α<sub>1</sub>, α<sub>2</sub>, α<sub>3</sub> and α<sub>6</sub>, but only very weakly with α<sub>5</sub>, we concentrated here on the C atom adjacent to the keto group in diazepam. We have previously shown that α<sub>1</sub>S205Cβ<sub>2</sub>γ<sub>2</sub> and α<sub>1</sub>T206Cβ<sub>2</sub>γ<sub>2</sub> receptors but not α<sub>1</sub>S204Cβ<sub>2</sub>γ<sub>2</sub> receptors interact covalently with 3-NCS [32]. Now homologous mutations were introduced into other α subunit isoforms.

We studied the covalent reaction at the level of radioactive ligand binding at receptors expressed in transfected HEK 293 cells and at the functional level characterizing chloride current mediated by receptors expressed in *Xenopus* oocytes. In general, we found very good agreement of the results obtained by the two strategies. In case covalent reaction occurs not in the binding pocket, but on the access pathway of 3-NCS, we would not expect an allosteric modulation of the corresponding receptor. Thus the agreement between the strategies can be taken as evidence for a reaction in the binding pocket.

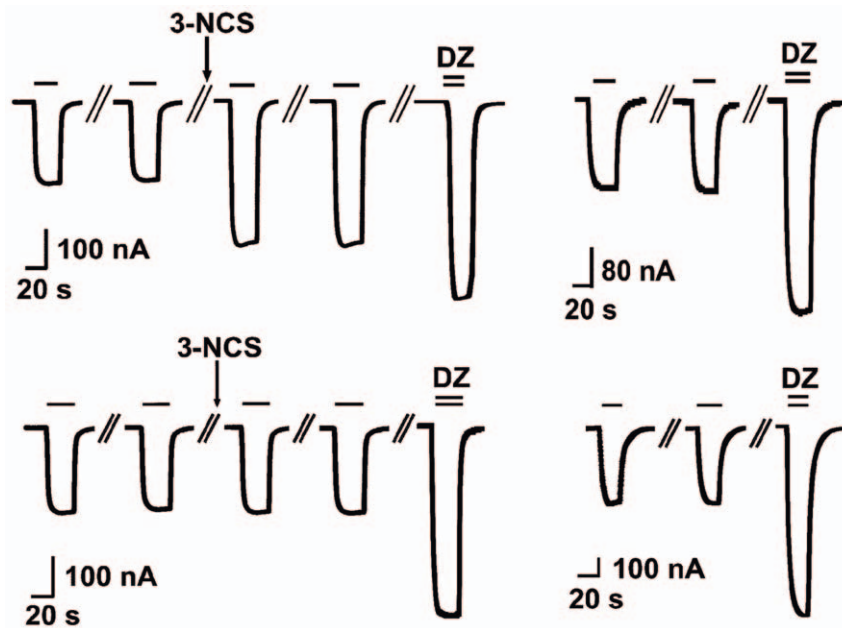
In the following we discuss in sequence our observations for the position homologous to α<sub>1</sub>S204, to α<sub>1</sub>S205, to α<sub>1</sub>T206 and to α<sub>1</sub>G207 in α<sub>x</sub>β<sub>2</sub>γ<sub>2</sub> (x = 1, 2, 3, 5, 6) receptors.

Position homologous to α<sub>1</sub>S204: At the binding level, mutated α<sub>2</sub>β<sub>2</sub>γ<sub>2</sub> and α<sub>5</sub>β<sub>1</sub>γ<sub>2</sub> showed covalent reaction, while α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> and α<sub>6</sub>β<sub>2</sub>γ<sub>2</sub> did not. At the functional level, exclusively mutated α<sub>5</sub>β<sub>2</sub>γ<sub>2</sub> showed covalent reaction. We have no explanation for the discrepancy concerning α<sub>2</sub>β<sub>2</sub>γ<sub>2</sub>. Possibly, 3-NCS acts as an antagonist here and covalent reaction at the α/β subunit interface promotes stimulation by diazepam. α<sub>3</sub>β<sub>2</sub>γ<sub>2</sub> could not be expressed at sufficient extent to determine reaction levels.

Position homologous to α<sub>1</sub>S205: At the binding level, mutated α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>, α<sub>2</sub>β<sub>2</sub>γ<sub>2</sub> and α<sub>5</sub>β<sub>1</sub>γ<sub>2</sub> showed covalent reaction, while mutated α<sub>6</sub>β<sub>2</sub>γ<sub>2</sub> did not. At the functional level, the same result was obtained. In addition, α<sub>3</sub>β<sub>2</sub>γ<sub>2</sub> might have reacted covalently at the functional level, but reaction was at the threshold for significance.

Position homologous to α<sub>1</sub>T206: At the binding level, all mutated receptors investigated α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>, α<sub>2</sub>β<sub>2</sub>γ<sub>2</sub>, α<sub>5</sub>β<sub>1</sub>γ<sub>2</sub> and α<sub>6</sub>β<sub>2</sub>γ<sub>2</sub> showed covalent reaction. At the functional level, the same result



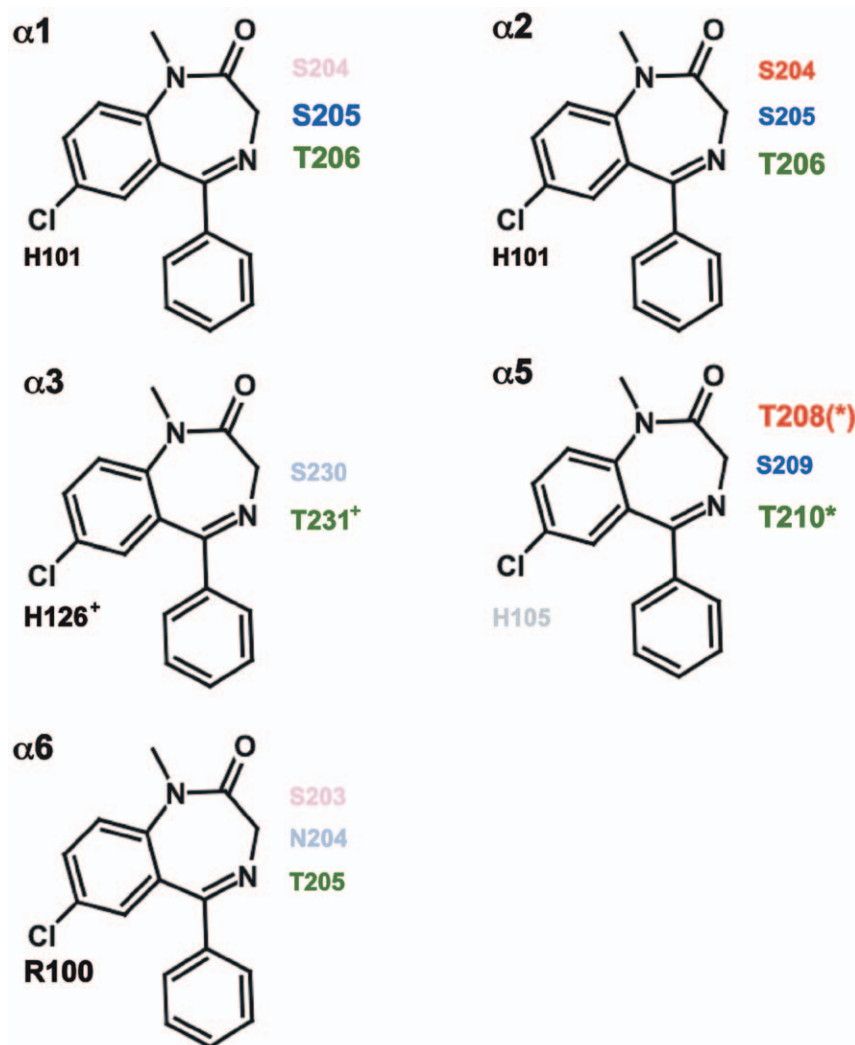


**Figure 5. Covalent reaction at the functional level.** Exposure of mutated  $\alpha_1S205C\beta_2\gamma_2$  receptors to 3-NCS results in a covalent reaction. a) Receptors were exposed twice to 0.5  $\mu$ M GABA followed by a washing period and a 3 min incubation in 10  $\mu$ M 3-NCS. After this treatment GABA was applied twice with a 3 min interval. In some experiments GABA was applied up to six times. Thus, the current elicited by GABA was stimulated irreversibly. Application of 0.5  $\mu$ M GABA in combination with 1  $\mu$ M diazepam results in a further stimulation of the partially irreversibly reacted receptors that was smaller than that seen upon b) direct exposure to diazepam in independent experiments. Exposure of  $\alpha_1T204C\beta_2\gamma_2$  receptors 3-NCS does not result in a covalent reaction. c) Receptors were exposed twice to 0.5  $\mu$ M GABA followed by a 3 min incubation in 10  $\mu$ M 3-NCS. Application of 0.5  $\mu$ M GABA in combination with 1  $\mu$ M diazepam results in stimulation similar to the stimulation seen in an oocyte that had not been previously exposed to 3-NCS (Fig. 4d).  
doi:10.1371/journal.pone.0042101.g005

**Table 2. Covalent reaction at the functional level.**

Mutated receptor	Mutation homologous to	DZ	n	3-NCS	p	3-NCS +DZ	n
$\alpha_1S204C\beta_2\gamma_2^+$		126 $\pm$ 9	3	4 $\pm$ 4	n.s.	115 $\pm$ 34	3
$\alpha_1S205C\beta_2\gamma_2$		113 $\pm$ 7	3	60 $\pm$ 43	<0.03	131 $\pm$ 32	3
$\alpha_1T206C\beta_2\gamma_2$		82 $\pm$ 18	6	119 $\pm$ 51	<0.01	204 $\pm$ 62	4
$\alpha_2S204C\beta_2\gamma_2^+$	$\alpha_1S204C$	215 $\pm$ 42	3	1 $\pm$ 10	n.s.	218 $\pm$ 30	3
$\alpha_2S205C\beta_2\gamma_2$	$\alpha_1S205C$	191 $\pm$ 53	3	35 $\pm$ 10	<0.01	177 $\pm$ 25	3
$\alpha_2T206C\beta_2\gamma_2$	$\alpha_1S206C$	148 $\pm$ 8	3	53 $\pm$ 29	<0.01	168 $\pm$ 54	3
$\alpha_3S229C\beta_2\gamma_2$	$\alpha_1S204C$	i.e.		i.e.		i.e.	
$\alpha_3S230C\beta_2\gamma_2$	$\alpha_1S205C$	149 $\pm$ 44	3	15 $\pm$ 11	n.s.	158 $\pm$ 44	3
$\alpha_3T231C\beta_2\gamma_2$	$\alpha_1S206C$	88 $\pm$ 45	3	28 $\pm$ 16	<0.03	165 $\pm$ 8	3
$\alpha_5T208C\beta_2\gamma_2$	$\alpha_1S204C$	129 $\pm$ 38	7	23 $\pm$ 18	<0.05	83 $\pm$ 25	5
$\alpha_5S209C\beta_2\gamma_2$	$\alpha_1S205C$	103 $\pm$ 3	3	46 $\pm$ 17	<0.01	140 $\pm$ 20	3
$\alpha_5T210C\beta_2\gamma_2$	$\alpha_1S206C$	82 $\pm$ 14	3	10 $\pm$ 5	n.s.	47 $\pm$ 8	3
$\alpha_6S203C\beta_2\gamma_2^+$	$\alpha_1S204C$	50 $\pm$ 20*	3	0 $\pm$ 2	n.s.	57 $\pm$ 21*	3
$\alpha_6N204C\beta_2\gamma_2^+$	$\alpha_1S205C$	70 $\pm$ 40*	3	-4 $\pm$ 6	n.s.	71 $\pm$ 40*	3
$\alpha_6T205C\beta_2\gamma_2$	$\alpha_1S206C$	48 $\pm$ 6*	3	14 $\pm$ 8	<0.05	58 $\pm$ 21*	3

Mutated GABA<sub>A</sub> receptors were expressed in *Xenopus* oocytes. Allosteric stimulation by 1  $\mu$ M diazepam was determined (column labelled DZ (diazepam)) at EC<sub>2-5</sub> for GABA. Data are given as % allosteric modulation. In independent experiments oocytes were exposed to GABA followed by 10  $\mu$ M 3-NCS and after removing non-covalently reacted 3-NCS, allosteric stimulation was determined (column labelled 3-NCS). Subsequently the same oocyte was exposed to 1  $\mu$ M diazepam (column labelled 3-NCS + diazepam) and allosteric stimulation was determined as compared to the initial application of GABA. \*1  $\mu$ M Abecarnil was used. i.e. low expression, expressed currents were too small for measurement of covalent effects. p was determined with the one-way ANOVA followed by a post-hoc Dunnett's test where the non-responsive receptors indicated with (\*) served as one of the samples (mean  $\pm$  mean SD, 0.25 $\pm$ 5.5, n=4). Data are given as mean  $\pm$  SD.  
doi:10.1371/journal.pone.0042101.t002



**Figure 6. Summary.** The results obtained with NCS [33] and the results obtained with 3-NCS described here are summarized. For each receptor isoform  $\alpha_x\beta_1\gamma_2$  ( $x = 1, 2, 3, 5, 6$ ) amino acid residues homologous to 101 in  $\alpha_1$  are shown in black, those homologous to 204 are shown in red, those homologous to 205 are shown in blue and those homologous to 206 are shown in green. Residue 101 is located near to the C atom carrying the Cl atom in diazepam, and 204, 205 and 206 are near to C atom adjacent to the keto group. The residues showing covalent reaction are shown in full color and those showing no reaction coloured in reduced saturation. The residues in larger font size react with a high apparent affinity. The residues marked with \* and (\*) indicate an antagonistic effect and a partial modulatory effect by 3-NCS on the corresponding  $\alpha_5\beta_2\gamma_2$  receptors, respectively. The residues marked with + indicate that the corresponding  $\alpha_3\beta_2\gamma_2$  receptors were only investigated at the functional level.  
 doi:10.1371/journal.pone.0042101.g006

was obtained except for possibly  $\alpha_6\beta_2\gamma_2$ , which is at the threshold for significance. In addition, mutated  $\alpha_3\beta_2\gamma_2$  reacted covalently.

Position homologous to  $\alpha_1$ G207: At the binding and at the functional level mutated  $\alpha_1\beta_2\gamma_2$  and  $\alpha_2\beta_2\gamma_2$  receptors were missing the benzodiazepine binding site. In functional experiments currents induced by GABA were not affected by the mutations. All other receptor isoforms were not tested.

In summary there is evidence for covalent reaction of the  $\alpha_1$  subunit in residues **205** and **206**; of the  $\alpha_2$  subunit in residues 204, 205 and **206**; of the  $\alpha_3$  subunit at least in residue 206 and possibly 205; of the  $\alpha_5$  subunit in residues **204**, 205 and **206**; and of the  $\alpha_6$  subunit exclusively in residue 206. The residues highlighted in bold face react with a high apparent affinity. It is interesting to note that the residue homologous to 206 reacts covalently in all receptors. Unexpectedly, the  $\alpha_2$  subunit showed a similar reactivity pattern as the  $\alpha_5$  subunit.

These observations should be combined with data on another region of the benzodiazepine binding pocket. The region of the –Cl atom in diazepam has previously been investigated in different GABA<sub>A</sub> receptor isoforms [33]. A molecule made reactive in this position interacted best with  $\alpha_6$  containing receptors and very little with  $\alpha_5$  containing receptors while the variants with  $\alpha_1$ ,  $\alpha_2$ , and  $\alpha_3$  were intermediate.

The combined set of data is visualized in Fig. 6. The size of the lettering of the residues correlates qualitatively with the affinity of the covalent reaction. Our observations will help in modelling of the benzodiazepine binding pocket in different  $\alpha$  subunit containing isoforms of the GABA<sub>A</sub> receptor.

## Acknowledgments

We thank Dr. V. Niggli for carefully reading the manuscript.

## Author Contributions

Conceived and designed the experiments: ES BPL. Performed the experiments: BPL RB. Analyzed the data: BPL ES. Contributed reagents/materials/analysis tools: MG. Wrote the paper: BPL ES.

## References

- Macdonald RL, Olsen RW (1994) GABA<sub>A</sub> receptor channels. *Annu Rev Neurosci* 17: 569–602.
- Rabow LE, Russek SJ, Farb DH (1995) From ion currents to genomic analysis: recent advances in GABA<sub>A</sub> receptor research. *Synapse* 21: 189–274.
- Sieghart W (1995) Structure and pharmacology of gamma-aminobutyric acidA receptor subtypes. *Pharmacol Rev* 47: 181–233.
- Sieghart W, Sperk G (2002) Subunit composition, distribution and function of GABA<sub>A</sub> receptor subtypes. *Curr Top Med Chem* 2: 795–816.
- Barnard EA, Skolnick P, Olsen RW, Möhler H, Sieghart W, et al. (1998) International Union of Pharmacology. XV. Subtypes of gamma-aminobutyric acid A receptors: classification on the basis of subunit structure and receptor function. *Pharmacol Rev* 50: 291–313.
- Olsen RW, Sieghart W (2008) International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. *Update Pharmacol Rev* 60: 243–60.
- McKernan RM, Whiting PJ (1996) Which GABA<sub>A</sub>-receptor subtypes really occur in the brain? *Trends Neurosci* 19: 139–143.
- Chang Y, Wang R, Barot S, Weiss DS (1996) Stoichiometry of a recombinant GABA<sub>A</sub> receptor. *J Neurosci* 16: 5415–5424.
- Tretter V, Ehya N, Fuchs K, Sieghart W (1997) Stoichiometry and assembly of a recombinant GABA<sub>A</sub> receptor subtype. *J Neurosci* 17: 2728–2737.
- Farrar SJ, Whiting PJ, Bonnert TP, McKernan RM (1999) Stoichiometry of a Ligand gated ion channel determined by fluorescence energy transfer. *J Biol Chem* 274: 10100–10104.
- Baumann SW, Baur R, Sigel E (2001) Subunit arrangement of gamma-aminobutyric acid type A receptors. *J Biol Chem* 276: 36275–36280.
- Baumann SW, Baur R, Sigel E (2002) Forced subunit assembly in  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub> receptors. Insight into the absolute arrangement. *J Biol Chem* 277: 46020–46025.
- Baur R, Minier F, Sigel E (2006) A GABA(A) receptor of defined subunit composition and positioning: concatenation of five subunits. *FEBS Lett* 580: 1616–1620.
- Sigel E, Bühr A (1997) The benzodiazepine binding site of GABA<sub>A</sub> receptors. *Trends Pharmacol Sci* 18: 425–429.
- Sigel E, Luscher BP (2011) A closer look at the high affinity binding site for benzodiazepines on GABA<sub>A</sub> receptors. *Curr Top Med Chem* 11: 241–246.
- Pöldl A, Hauer B, Fuchs K, Tretter V, Sieghart W (2003) Subunit composition and quantitative importance of GABA<sub>A</sub> receptor subtypes in the cerebellum of mouse and rat. *J Neurochem* 87: 1444–1455.
- Minier F, Sigel E (2004) Positioning of the  $\alpha$ -subunit isoforms confers a functional signature to  $\gamma$ -aminobutyric acid type A receptors. *Proc Natl Acad Sci U S A* 101: 7769–7774.
- Wieland HA, Lüddens H, Seeburg PH (1992) A single histidine in GABA<sub>A</sub> receptors is essential for benzodiazepine agonist binding. *J Biol Chem* 267: 1426–1429.
- Benson JA, Low K, Keist R, Möhler H, Rudolph U (1998) Pharmacology of recombinant gamma-aminobutyric acidA receptors rendered diazepam-insensitive by point-mutated alpha-subunits. *FEBS Lett* 431: 400–404.
- Davies PA, Hanna MC, Hales TG, Kirkness EF (1997) Insensitivity to anaesthetic agents conferred by a class of GABA(A) receptor subunit. *Nature* 385: 820–823.
- Dunn SMJ, Davies M, Muntoni AL, Lambert JJ (1999) Mutagenesis of the rat  $\alpha 1$  subunit of the  $\gamma$ -amino butyric acid type A receptor reveals the importance of residue 101 in determining the allosteric effects of benzodiazepine site ligands. *Mol Pharmacol* 56: 768–774.
- Rudolf U, Crestani F, Benke J, Brünig I, Benson JA, et al. (1999) Benzodiazepine actions mediated by specific  $\mu$ -aminobutyric acid<sub>A</sub> receptor subtypes. *Nature* 401: 796–800.
- McKernan RM, Rosahl TW, Reynolds DS, Sur C, Wafford KA, et al. (2000) Sedative but not anxiolytic properties of benzodiazepines are mediated by the GABA(A) receptor alpha1 subtype. *Nature Neurosci* 3: 587–592.
- Löw K, Crestani F, Keist R, Benke D, Brünig I, et al. (2000) Molecular and Neuronal Substrate for the Selective Attenuation of Anxiety. *Science* 290: 131–134.
- Crestani F, Löw K, Keist R, Mandelli M-J, Möhler H, et al. (2001) Molecular targets for the myorelaxant action of diazepam. *Mol Pharmacol* 59: 442–445.
- Crestani F, Keist R, Fritschy J-M, Benke D, Vogt K, et al. (2002) Traces fear conditioning involves hippocampal alpha 5 GABA<sub>A</sub> receptors. *Proc Natl Acad Sci U S A* 99: 8980–8985.
- Dias R, Sheppard WF, Fradley RL, Garrett EM, Stanley JL, et al. (2005) Evidence for a significant role of alpha 3-containing GABA<sub>A</sub> receptors in mediating the anxiolytic effects of benzodiazepines. *J Neurosci* 25: 10682–8.
- Berezhnoy D, Nyfeler Y, Gonthier A, Schwob H, Goeldner M, et al. (2004) Towards a relative orientation of benzodiazepines in their binding pocket on GABA<sub>A</sub> receptors. *J Biol Chem* 279: 3160–3168.
- Berezhnoy D, Baur R, Gonthier A, Foucaud B, Goeldner M, et al. (2005) Conformational changes at the benzodiazepine binding site of GABA<sub>A</sub> receptors detected with a novel technique. *J Neurochem* 92: 859–866.
- Tan KR, Gonthier A, Baur R, Ernst M, Goeldner M, et al. (2007a) Proximity-accelerated chemical coupling reaction in the benzodiazepine binding site of GABA<sub>A</sub> receptors: superposition of different allosteric modulators. *J Biol Chem* 282: 26316–26325.
- Tan KR, Baur R, Gonthier A, Goeldner M, Sigel E (2007b) Two neighboring residues of loop A of the  $\alpha 1$  subunit point towards the benzodiazepine binding site of GABA<sub>A</sub> receptors. *FEBS Lett* 581: 4718–4722.
- Tan KR, Baur R, Charon S, Goeldner M, Sigel E (2009) Relative positioning of diazepam in the benzodiazepine binding pocket of GABA<sub>A</sub> receptors. *J Neurochem* 111: 1264–1273.
- Baur R, Tan KR, Lüscher BP, Gonthier A, Goeldner M, et al. (2008) Covalent modification of GABA<sub>A</sub> receptor isoforms by a diazepam analogue provides evidence for a novel benzodiazepine binding site that prevents modulation by these drugs. *J Neurochem* 106: 2353–2356.
- Walters RJ, Hadley SH, Morris KDW, Amin J (2000) Benzodiazepines act on GABA<sub>A</sub> receptors via two distinct and separable mechanisms. *Nature Neurosci* 3: 1274–1231.
- Ramerstorfer J, Furtmüller R, Sarto-Jackson I, Varagic Z, Sieghart W, et al. (2011) The GABA<sub>A</sub> receptor Alpha+beta- interface: a novel target for subtype selective drugs. *J Neurosci* 31: 870–877.
- Bertocci B, Miggiano V, Da Prada M, Dembic Z, Lahm H-W, et al. (1991) Human catechol-O-methyltransferase: cloning and expression of the membrane-associated form. *Proc Natl Acad Sci U S A* 88: 1416–1420.
- Chen C, Okayama H (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* 7: 2745–2752.
- Sigel E (1987) Properties of single sodium channels translated by *Xenopus* oocytes after injection with messenger ribonucleic acid. *J Physiol* 386: 73–90.
- Sigel E, Minier F (2005) The *Xenopus* oocyte: system for the study of functional expression and modulation of proteins. *Mol Nutr Food Res* 49: 228–234.
- Boileau AJ, Baur R, Sharkey LM, Sigel E, Czajkowski C (2002) The relative amount of cRNA coding for gamma2 subunits affects stimulation by benzodiazepines in GABA(A) receptors expressed in *Xenopus* oocytes. *Neuropharmacology* 43: 695–700.
- Foucaud B, Perret P, Grutter T, Goeldner M (2001) Cysteine mutants as chemical sensors for ligand-receptor interactions *Trends Pharmacol Sci* 22: 170–173.